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LAY, A New Endoplasmic Reticulum Retention Signal for UBIAD1 Protein

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Abstract: *In this paper, we observed the co-localization of UBIAD1 and Ds Red-ER (an ER marker) in bladder carcinoma cell line T24 and human embryonic kidney cell line HEK293T. UBIAD1 accumulated on the endoplasmic reticulum (ER) in both T24 and HEK293T cell lines. Site-directed mutagenesis defined a LAY motif, positioned on 71-73 amino acids of the UBIAD1 N-terminus, as the ER retention signal of UBIAD1. Based upon flow cytometry analysis, it is shown that mutations of LAY motif increased the UBIAD1-induced apoptosis of T24 cells. Disruption of the UBIAD1 localization on ER influenced its tumor suppressant activity.*

Keywords: *UBIAD1, endoplasmic reticulum, co-localization, retention signal, HEK293T.*

1. INTRODUCTION

Ubiad1 (UbiA prenyltransferase domain containing 1), also known as *Tere1* (transitional epithelial response gene), was first cloned as a tumor suppressor gene for human bladder carcinoma [1, 2]. Later on, mutations in Ubiad1 were diagnosed as a cause of Schnyder corneal dystrophy (SCD). SCD is a rare autosomal dominant disease characterized by an abnormal increase in cholesterol and phospholipid deposition in the cornea, resulting in progressive corneal opacification and visual loss [3, 4, 5, 6]. UBIAD1 protein has also been shown to physically interact with apolipoprotein E and can lower the intracellular cholesterol level in HEK293 cells [7, 8]. Additionally, UBIAD1 has been recently characterized as the first enzyme responsible for human vitamin K biosynthesis [9], in which UBIAD1 participates in the conversion of vitamin K derivatives into menaquinone 4 (MK-4). Vos et al. reported that *Drosophila ubiad1/heix* is a modifier of *pink1*, a gene mutated in Parkinson's disease that affects mitochondrial function [10]. Recently, Mugoniet al. [11] showed that UBIAD1 is a prenyltransferase required for CoQ10 biosynthesis at Golgi membranes, protecting cardiovascular system from reactive oxygen species (ROS) by regulating eNOS activity.

Our previous research has demonstrated that UBIAD1 is a negative regulator of the Ras-MAPK signaling pathway [12]. Knocking down UBIAD1 expression with siRNA directly activates Ras-MAPK signal transduction pathway, resulting in up-regulation of hTERT transcription and ultimately cell proliferation. Study on the *Drosophila heix* gene, which encodes an ortholog of human UBIAD1, has found a correlation between the malignant blood tumor phenotype and an increased number of *Drosophila* blood cells carrying mutant non-functional *heix*. This is consistent with the role of UBIAD1 being a bona fide tumor suppressor [1, 12, 13].

Studies on the subcellular localization of the wild type and the mutant human UBIAD1 found that neither the wild type nor the mutant UBIAD1 colocalizes with the ER inside cultured human keratocytes [6]. On the other hand, N102S mutant UBIAD1 did colocalize with a mitochondrial marker (OXPHOS complex I, NADH dehydrogenase) in keratocytes derived from the SCD family. In addition, Nakagawa et al. showed that UBIAD1-GFP colocalized with the endoplasmic reticulum marker (ER-tracker Red), but not with the Golgi marker (BODIPY-TR ceramide) in human osteoblast-like MG-63 cells [9]. *Drosophila* UBIAD1/*Heix* expressed in S2 cells localized

to the mitochondria just like in human keratocytes [6, 10]. Mugoni et al. [11] showed that in human endothelial cells, UBIAD1 is localized on the Golgi membrane. We have previously reported that UBIAD1 is colocalized with the Golgi in human bladder carcinoma cell line T24 and a RPWS motif has been identified as the Golgi retention signal of UBIAD1[14]. Using a combination of biochemical and cellular approaches, we found that UBIAD1 accumulates on both Golgi and ER in human bladder carcinoma cell line T24. In this paper, we explored the retention signal of UBIAD1 on ER and found out that the ER retention signal of UBIAD1 is a novel protein motif, LAY, which affects the tumor suppressant activity of UBIAD1.

2. MATERIAL AND METHODS

2.1. Plasmids, Antibodies and Reagents

The plasmid pOTB7-UBIAD1 was purchased from Open Biosystems (USA). The enhanced green fluorescent protein (EGFP) vector plasmid EGFP-N1 was purchased from Invitrogen (USA). The full-length UBIAD1 cDNA was subcloned into plasmid EGFP-N1 to make a pUBIAD1-EGFP construct. The plasmid DsRed2 used to express red fluorescent protein was obtained from Clontech (USA). As to the plasmid Ds Red-ER, an endoplasmic reticulum (ER) targeting sequence of calreticulin was fused to the 5' end of DsRed2 and the ER retention sequence, KDEL, was fused to the 3' end of DsRed2. Plasmid containing orail1 gene, which encodes an essential pore subunit of the CRAC channel on the plasmamembrane, was kindly provided by Professor Ding JP at Key Laboratory of Molecular Biophysics, Huazhong University of Science and Technology (Wuhan, China). Orail1-EGFP was constructed by fusing the EGFP at the C-terminus of Orail1. UBIAD1-N-Orail1-EGFP was constructed by fusing the UBIAD1 N-terminus (the first 80 amino acids) to the N-terminus of Orail1-EGFP. Plasmid Flag-UBIAD1 was tagged by the Flag epitope (DYKDDDDK) at the N-terminus of UBIAD1. Plasmid UBIAD1-102Myc was constructed by inserting c-Myc epitope at the amino acid 102 of the UBIAD1 protein. To construct UBIAD1-Myc plasmid, c-Myc epitope was fused at the C-terminus of UBIAD1.

Plasmids UBIAD1(1-2)-EGFP, UBIAD1(2-2)-EGFP, UBIAD1(3-2)-EGFP, UBIAD1(4-2)-EGFP, UBIAD1(2-1)-EGFP, UBIAD1(3-1)-EGFP and UBIAD1(4-1)-EGFP were constructed by fusing part of UBIAD1 at the 5' end of EGFP. A series of UBIAD1 deletion clones (20AA, 40AA, 50AA, 55AA, 60AA, 65AA, 70AA and 75AA) were made by deleting 20, 40, 50, 55, 60 , 65 , 70 and 75 amino acids from the N-terminus of UBIAD1 and subcloned into the 5' end of plasmid EGFP-N1. Based on the PCR site-directed mutagenesis, a series of UBIAD1-EGFP subclones with specific amino acid mutations were constructed. The 71-75 amino acids (LAYRS) of UBIAD1 protein was subsequently mutated into AAAAA, AAARS, LAYAA, AAYRS, LGYRS, LRYRS, LAAYRS, LAYAS and LAYRA, respectively.

Anti-c-Myc monoclone antibody and anti-Flag monoclone antibody were obtained from Proteintech (USA). Anti-UBIAD1 primary antibody was purchased from Abcam (USA).

Cycloheximide was used at a final concentration of 100μg/ml. After being transfected for 24 hours, HEK293T cells were then treated by cycloheximide for 4.5 hrs, 5 hrs, 10 hrs, and 20 hrs, respectively.

2.2. Cell Culture and Transfection

Human embryonic kidney cell line HEK293T and human bladder carcinoma cell line T24 were purchased from China Center for Type Culture Collection (Wuhan, CCTCC). HEK293T cells were maintained in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). T24 cells were raised in Minimum Essential Medium with Eagle's salts (MEM) supplemented with 10% fetal bovine serum (FBS). HEK293T and T24 cells were all cultured in CO_2 incubator at 37℃(5% CO_2).

All DNA transfection in human cell lines were performed by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Prior to the transfection, cells were transferred into 6-well or 12-well plates without antibiotics and cultured overnight.

2.3. Confocal Microscopy

For immunofluorescence(IF), transfected cells were fixed with 4% paraformaldehyde(PF) in PBS for 15 min followed by permeabilization with 0.3% Triton X-100 (PBST) for 20 min. Cells were then washed with PBS (pH 7.2) and blocked with 2% BSA in PBS. Anti-c-Myc monoclonal antibody, anti-Flag monoclonal antibody and anti-UBIAD1 primary antibody were all used at 1:1000 in PBS 2% BSA. Similarly, goat anti-mouse and goat anti-rabbit FITC-conjugated secondary antiserum (green) were used at 1:1000 in PBS 2% BSA. The nucleus was revealed by 496-diamidino-2-phenylindole (DAPI) staining.

A confocal system (FV500, Olympus) and an inverted fluorescent microscopy set (IX81, Olympus) have been used in order to detect fluorescent signals. FITC and EGFP were excited by a 488 nm argon ion laser. Ds Red-ER was excited by a 543 nm He-Ne laser. DAPI was excited by a 405 nm diode laser. FLUOVIEW (Olympus) was used as the image acquisition software. Images were acquired and assembled with ImageJ (National Institutes of Health, USA Public Domain) and CorelDRAW X3.

2.4. Flow Cytometry Assay

Bladder carcinoma T24 cells were transfected with wild type and mutant (LAYRS→AAAAA) UBIAD1, then fixed with 70% ethanol overnight and stained with PI 48 hr later. A Flow Cytometer (FC500) was exploited for cell count.

3. RESULTS AND DISCUSSION

3.1. As a Membrane Protein, UBIAD1 is Localized in the Juxta-nuclear Area of the Cytosol

To study its subcellular localization, UBIAD1 2-D model embedded in lipid membrane was generated using the bioinformatics software TOPCONS. UBIAD1 is a putative nine transmembrane protein with its N-terminus and C-terminus located on the opposite side of the lipid membrane (Figure 1A). In order to track UBIAD1, we generated a construct (UBIAD1-EGFP) encoding for a fusion protein consisting of UBIAD1 tagged at its carboxyl terminus with an enhanced green fluorescent protein (EGFP). The UBIAD1-EGFP construct was then used to transfect HEK293T and bladder carcinoma T24 cells (Figure 1B). Figure 1B clearly shows the affluent expression of UBIAD1-EGFP around the juxta-nuclear area inside the cytosol, for both cultured HEK293T and bladder carcinoma T24 cell lines (Figure 1B, panel 2). However, T24 cells were not suitable neither for subcellular localization study nor for intracellular trafficking of UBIAD1 because of two reasons: the low transfection efficiency the cells showed, and the growth inhibition caused by UBIAD1 [1]. Thus, human embryonic kidney cell line HEK293T was chosen due to its similar expression of UBIAD1 and higher transfection efficiency. The plasmid (Ds Red-ER) expressing the ER marker was transfected in both HEK293T and T24 cells (Figure 1B, panel 1). In both cell lines, UBIAD1 proteins were colocalized with ER marker, indicating that at least a portion of the UBIAD1 protein is localized on the ER in human bladder carcinoma cells and HEK293T cells (Figure 1B). To further explore the localization of UBIAD1 N-terminus and C-terminus, three tagged-proteins Flag-UBIAD1, UBIAD1-102Myc and UBIAD1-Myc were constructed (Figure1C). We overexpressed the tagged-proteins Flag-UBIAD1, UBIAD1-102Myc and UBIAD1-Myc in HEK293T cells to confirm that UBIAD1 is mainly distributed in the cytosol. These tags were detected using the corresponding first antibody and FITC-conjugated secondary antibody. When tagged with Flag epitope at the N-terminus of UBIAD1, Flag-UBIAD1 was not detected in HEK293T cells under non-permeabilized conditions (Figure 1D, a2, panel 2). However, under permeabilized conditions patchy aggregations of UBIAD1 were observed inside the cytosol of the transfected HEK293T cells, indicating the UBIAD1 N-terminus was found inside the cytosol (Figure 1D, a1, panel 2). Similar results were obtained when UBIAD1 was tagged with a c-Myc epitope at its C-terminus (Figure 1D, c1 and c2). This indicated that the C-terminus of UBIAD1 was found inside the cytosol. The fact that both N-terminus and C-terminus of UBIAD1 were inside the cytosol is consistent with previously mentioned UBIAD1-EGFP results (Figure 1B).

In b1 and b2 of Figure 1D, HEK293T cells were transfected with plasmid UBIAD1-102Myc (a c-Myc epitope inserted at amino acid 102 of UBIAD1). Consistent with the above observations, under non-permeabilized conditions, UBIAD1 was not detected using anti-c-Myc monoclone

antibody (Figure 1D, b2, panel 2). Under permeabilized conditions, cytoplasmic localization of UBIAD1 was observed (Figure 1D, b1, panel 2). Therefore, our results suggest that UBIAD1 is a membrane protein inside the cytoplasm, not on the plasma membrane.

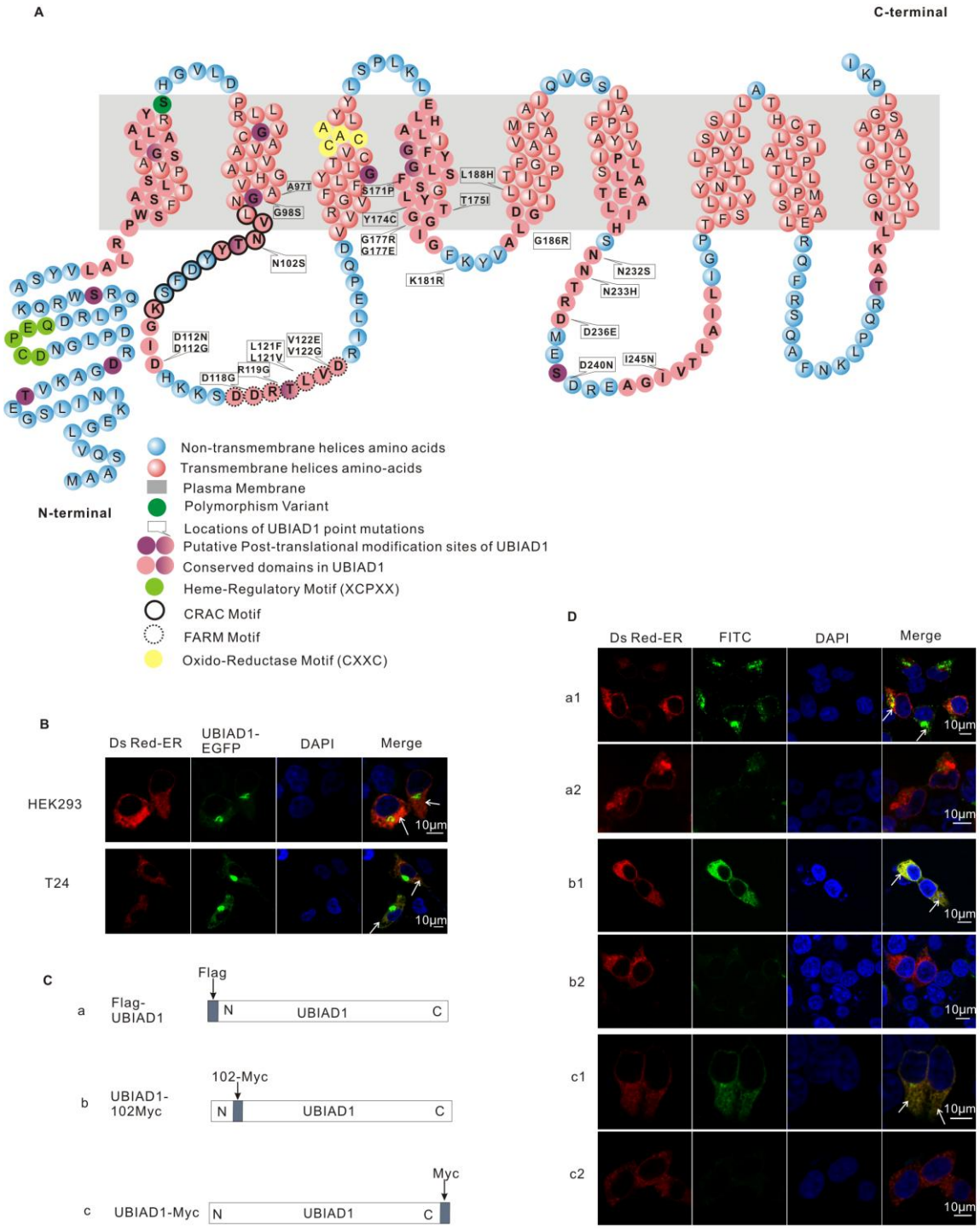

Fig1. *UBIAD1 is accumulated in the juxta-nuclear area inside HEK293T and T24 cells.*

(A)*UBIAD1 is a putative nine transmembrane protein. (**B**) Laser scanning confocal microscopic images of UBIAD1-EGFP (green) and Ds Red-ER (red). The merged images (yellow) indicate co-localization of UBIAD1 and ER both in the HEK293T and bladder carcinoma T24 cells. DAPI (blue) was used to mark the nuclei. (**C**) **a,** UBIAD1 was tagged with flag-epitope in N-terminus to make the tagged-protein Flag-UBIAD1; **b,** UBIAD1 was tagged with c-Myc-epitope at amino acid 102 to generate the UBIAD1-102myc; **c,** UBIAD1 was tagged with c-Myc-epitope at C-terminus to generate the UBIAD1-Myc. (**D**) UBIAD1 is located inside cytosol. The HEK293T cells were transfected by plasmid Flag-UBIAD1 (a1, a2), plasmid UBIAD1-102Myc (b1, b2) and plasmid UBIAD1-Myc (c1, c2), respectively. C-Myc antibody and Flag antibody were used to stain the transfected HEK293T cells under permeabilized (a1, b1 and c1, panel 2) and non-permeabilized (a2, b2 and c2, panel 2) conditions. Ds Red-ER was used to mark the ER (panel 1).*

3.2. UBIAD1 is Accumulated on the Endoplasmic Reticulum (ER) in HEK293T Cells

The previous data suggest that UBIAD1 might be localized on the ER. To explore this possibility, we overexpressed UBIAD1-EGFP in HEK293T cells, using Ds Red-ER to mark ER as before. Confocal microscopy images show that overexpressed UBIAD1 is accumulated at both ER and Golgi outside the nucleus (Figure 2A).

To eliminate the possibility that the ER localization of UBIAD1 in HEK293T cells was an artifact of protein overexpression, subcellular localization of the endogenous UBIAD1 was studied using the UBIAD1 primary antibody (Figure 2B). It is clear that the endogenous UBIAD1 colocalized with the ER marker (Figure 2B, h). Endogenous UBIAD1 is therefore localized on the ER.

In our previous report, we examined the subcellular localization of UBIAD1 in several cell lines including human bladder carcinoma cell line T24 and human prostate cancer cell line PC-3. The conclusion that UBIAD1 is a transmembrane protein which distributes on the endoplasmic reticulum (ER) and the Golgi apparatus in human bladder carcinoma cells has been given. The RPWS, a novel Golgi retention signal, was identified [2]. There was a question that why UBIAD1 could accumulate on both the Golgi and the ER. Since the Golgi retention signal has been characterized, we want to figure out whether part of UBIAD1 could retain it to the ER and whether there is a special signal for UBIAD1 to remain in the ER. In this paper, we came to the conclusion that there does exist a co-localization between the UBIAD1 protein and the Ds Red-ER (the ER marker) in HEK293T and T24 cells.

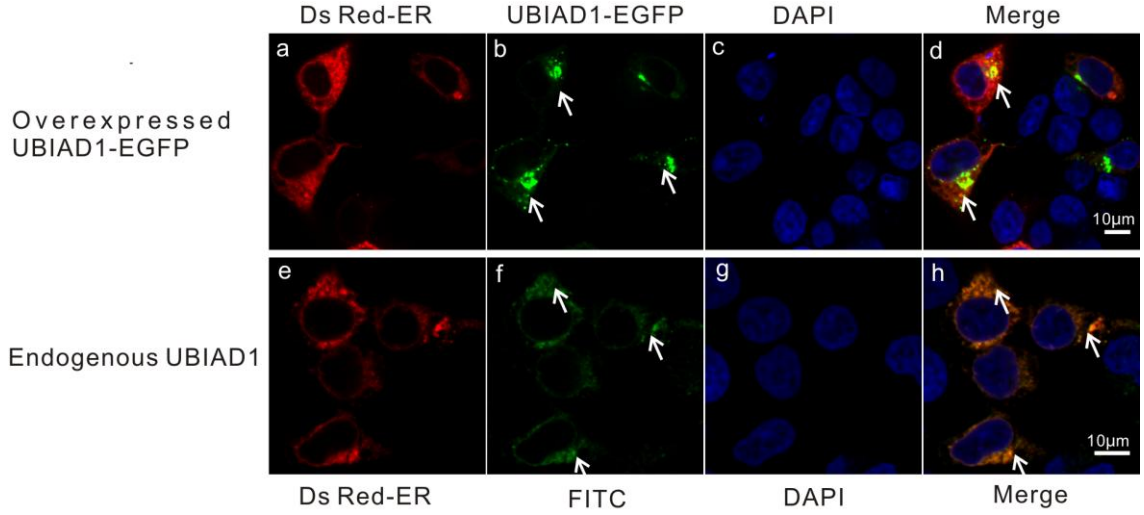

Fig2. *UBIAD1 is localized on the ER in HEK293T cell.*

a *and* ***e****, Ds Red-ER marker;* ***b****, Confocal microscopy of HEK293T cells overexpressing UBIAD1-EGFP;* ***f****, Confocal microscopy of HEK293T cells stained for endogenous UBIAD1 with UBIAD1 antibody and FITC-conjugated secondary antibody;* ***c*** *and* ***g****, DAPI staining;* ***d****, the merge of a, b and c;* ***h****, the merge of e, f and g.*

3.3. The N-Terminus of UBIAD1 is Crucial for its ER Retention

To study which sequence influences the retention of UBIAD1 on the ER, a series of subclones were constructed by truncating the full length of UBIAD1-EGFP (Figure 3A). As is shown in Figure 3A, plasmid UBIAD1(1-2)-EGFP, UBIAD1(2-2)-EGFP, UBIAD1(3-2)-EGFP and UBIAD1(4-2)-EGFP were constructed by deleting the N-terminus (the first 80 amino acids or more) of UBIAD1. Plasmid UBIAD1 (2-1)-EGFP, UBIAD1 (3-1)-EGFP and UBIAD1 (4-1)-EGFP were constructed by keeping the N-terminus and the first three transmembrane segments. These subclones were transfected into HEK293T cells and their subcellular localizations were examined with confocal microscopy. Deletion of the UBIAD1 N-terminus (the first 80 amino acids or more) resulted in diffusion of UBIAD1 across the entire HEK293T cells (Figure 3B). In Figure 3C, confocal microscopy images show that mutant proteins of UBIAD1-EGFP keeping the N-terminus are accumulated at ER area outside the nucleus. Summarizing results in figure 3B and figure 3C, localization of mutant UBIAD1 protein on the ER depends on whether it contains N-terminal sequence. UBIAD1(1-2)-EGFP and UBIAD1(2-1)-EGFP, two key UBIAD1-EGFP

mutant subclones, were also overexpressed in bladder carcinoma T24 cells (Figure 3D). Its confocal microscopy images reveal that the subcellular localization of these mutant UBIAD1 proteins in T24 cells is similar to those in the HEK293T cells, indicating that the N-terminus of UBIAD1 plays pivotal role in the ER localization of UBIAD1 in both T24 and HEK293T cells. The results of HEK293T cells are also applicable to the T24 cells. Therefore, the key sequence of ER retention for UBIAD1 seems to be within its N-terminus.

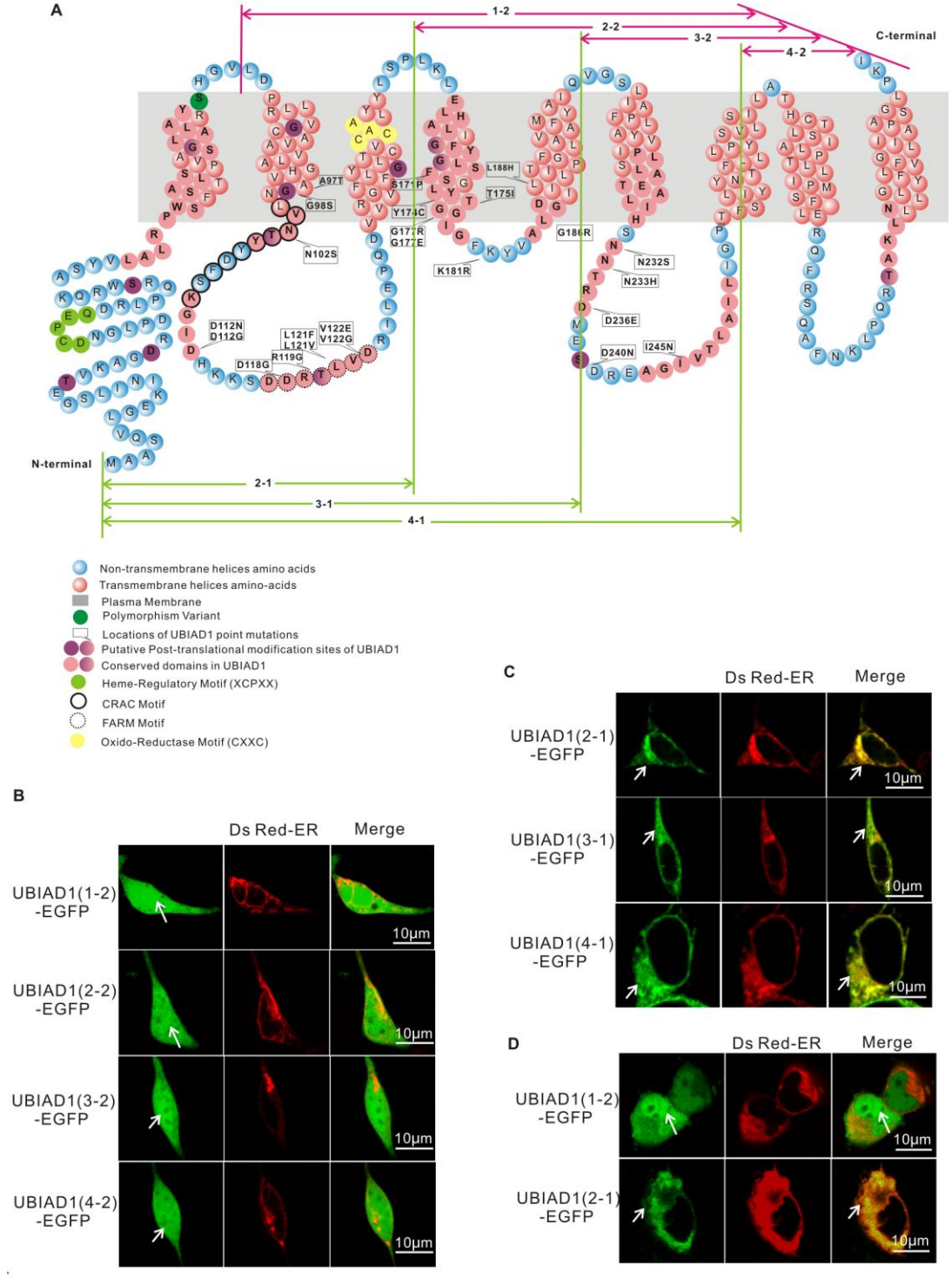

Fig3. *The N-terminus of UBIAD1 is crucial for its retention on the ER.*

(A)*Schematic representation of the secondary structure of UBIAD1 and the subclones of UBIAD1-EGFP; (**B**), Confocal microscopy of HEK293T cells overexpressing UBIAD1-EGFP subclones with N-terminus deleted (1-2, 2-2, 3-2, 4-2); (**C**) Confocal microscopy of HEK293T cells overexpressing UBIAD1-EGFP subclones with N-terminus remaining (2-1, 3-1, 4-1); (**D**) Confocal microscopy of T24 cells overexpressing UBIAD1-EGFP subclones 1-2 and 2-1, respectively.*

3.4. LAY, the ER Retention Signal of UBIAD1

Based on previous experiments, the N-terminus of UBIAD1 is necessary for its localization on the ER. In order to further characterize the specific amino acids (motif), which were crucial for the subcellular localization of UBIAD1, a series of deletion clones derived from full-length UBIAD1-EGFP were constructed by sequentially deleting the N-terminus (Figure 4A). For example, 20AA refers to the UBIAD1-EGFP deletion clone that the first 20 amino acids of UBIAD1 N-terminus were deleted. We transfected these deletion clones into HEK 293T cells as before. As the confocal images showed, there was no significant effect on the ER localization of UBIAD1 if only the first 70 amino acids were deleted (Figure 4C). Similar to the wild type UBIAD1 protein (WT), the mutant UBIAD1 protein with the first 20 amino acids deleted (20AA), the first 40 amino acids deleted (40AA), the first 50 amino acids deleted (50AA), the first 55 amino acids deleted (55AA), the first 60 amino acids deleted (60AA), the first 65 amino acids deleted (65AA) and the first 70 amino acids deleted (70AA) can all accumulate on the ER. But when we overexpressed the mutant UBIAD1 protein with the first 75 amino acids deleted (75AA) in HEK293T cells, the green fluorescence referring to the mutant UBIAD1 was diffused in cells and no longer gathered on the ER. Deleting the first 75 amino acids seems to disrupt the ER retention signal, resulting in diffusion of mutant UBIAD1 in HEK293T cells. To test and verify whether 71-75 amino acids (LAYRS) in N-terminus could affect the ER localization of UBIAD1, PCR-based site-directed mutagenesis was used to create a series of UBIAD1-EGFP mutant clones. The 71-75 amino acids (LAYRS) in N-terminus was sequentially mutated to AAAAA(71-75), AAARS(71-73), LAYAA(74-75), AAYRS(71A), LGYRS(72G), LAARS(73A), LAYAS(74A) and LAYRA(75A). We overexpressed these mutant UBIAD1-EGFP in HEK293T cells and confocal images were shown in Figure 5A (panel 2). The UBIAD1 mutants 71-75 and 71-73 can no longer concentrate on the ER and were diffused in HEK293T cells. Compared to the images of wild type UBIAD1 (WT), these mutant proteins (71-75, 71-73) can no longer co-localize with ER but rather spread across the whole cell, indicating the disruption of the ER localization. In contrast, confocal images of mutants 74-75, 71A, 72G, 73A, 74A and 75A were similar to that of wild type UBIAD1 (WT), indicating that these mutations do not disrupt the ER localization of UBIAD1. The wild type (WT) and some of the mutant UBIAD1-EGFP proteins were also expressed in bladder carcinoma T24 cells. Similar results were presented in Figure 5B. Therefore, at least part of the UBIAD1 ER retention signal lies within the amino acids 71-73 of the UBIAD1 N-terminus.

In Figure 4B, we showed the multiple alignment of the N-terminus of UBIAD1 from different species. Multiple alignment of the UBIAD1 N-terminus amino acid sequences from thirteen different species (giant panda, cattle, dog, zebrafish, chicken, human, willow ptarmigan, African elephant, Rhesus monkey, mouse, chimpanzee, norway rat and western clawed frog) showed that the L71-A72-Y73 motif is conserved across all thirteen species (Figure 4B). A tertiary model of the UBIAD1 N-terminus was predicted using the bioinformatics program Rosetta 3.1 (Figure 5C). Based on this prediction, the L71-A72-Y73 chain seems to serve as a unique protein motif. In conclusion, the 71-73 amino acids LAY motif are crucial to the ER localization of UBIAD1.

We also tested whether the N-terminus of UBIAD1 can target a membrane protein to the ER. For this purpose, Orail1, an essential pore subunit of the ion channel CRAC [15], was utilized. Orail1 is specifically localized on the plasma membrane, as shown by the confocal images of Orail1-EGFP (Figure 4D, line 1, panel 2). When the N-terminus of UBIAD1 was fused with Orail1-EGFP, the fusion protein UBIAD1-N-Orail1-EGFP can be targeted to the ER (Figure 4D, line 2, panel 2). However, when the 71-75 amino acids LAYRS mutated to AAAAA, the fusion protein UBIAD1-N (71-75)-Orail1-EGFP can no more be targeted to the ER (Figure 4D, line 3). The result indicates that the N-terminus of UBIAD1could provide the information necessary for UBIAD1 to be localized on the ER and the 71-75 amino acids, LAYRS, are essential for the localization.

Multiple alignment of the UBIAD1 N-terminus across thirteen species showed that the L71-A72-Y73 is extremely conservative. But if we only mutated the Leucine(L71), the Glycine(A72) or the Tyrosine(Y73) separately, we did not found that these mutations could influence the ER localization of UBIAD1. Then a question came to us: why the mutation of a single amino acid did not make any difference? In fact, UBIAD1 protein would diffuse to the whole cells when we

overexpressed the UBIAD1 (71-73 mutated) *in vitro*. Depending on the prediction of protein tertiary structure, we discovered that LAY is in a flexible area between helix and turn (Figure 5C). The Leucine (71L) belongs to nonpolar amino acid and Tyrosine (Y73) belongs to polar amino acid. This reminds us that the LAY is in a changeable environment and its structure can switch between binding to and dissociation with the receptor on the ER depending on specific conditions. So it is reasonable that there are parts of UBIAD1 distributed on the Golgi apparatus and the other on the ER.

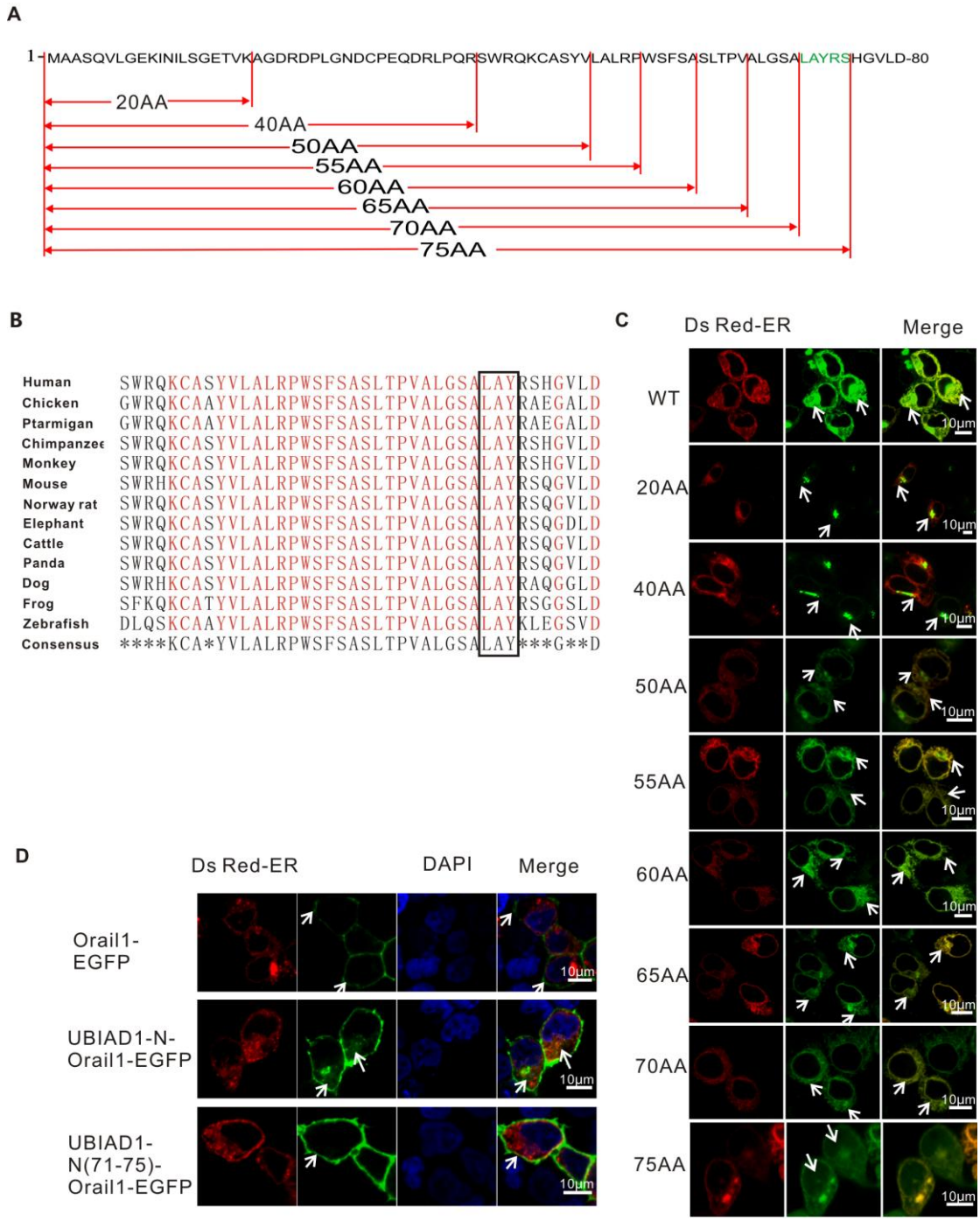

Fig4. *The 71-75 amino acids in N-terminus are the most crucial amino acids for the retention of UBIAD1 on ER.*

(A)*A series of deletion clones were constructed for UBIAD1-EGFP (i.e., 20AA, the first 20 amino acids of UBIAD1 N-terminus was deleted);* ***(B)*** *Multiple alignment of the N-terminus of the UBIAD1 orthologs from thirteen different species using program Geneious. LAY (71-73) is conserved across all ten species;* ***(C)*** *Confocal microscopy of HEK293T cells overexpressing UBIAD1-EGFP deletion clones shown in A (WT, wild type UBIAD1-EGFP);* ***(D)*** *Confocal microscopy of HEK293T cells overexpressing Orail1-EGFP, UBIAD1-N-Orail1-EGFP and UBIAD1-N(71-75)-Orail-EGFP respectively.*

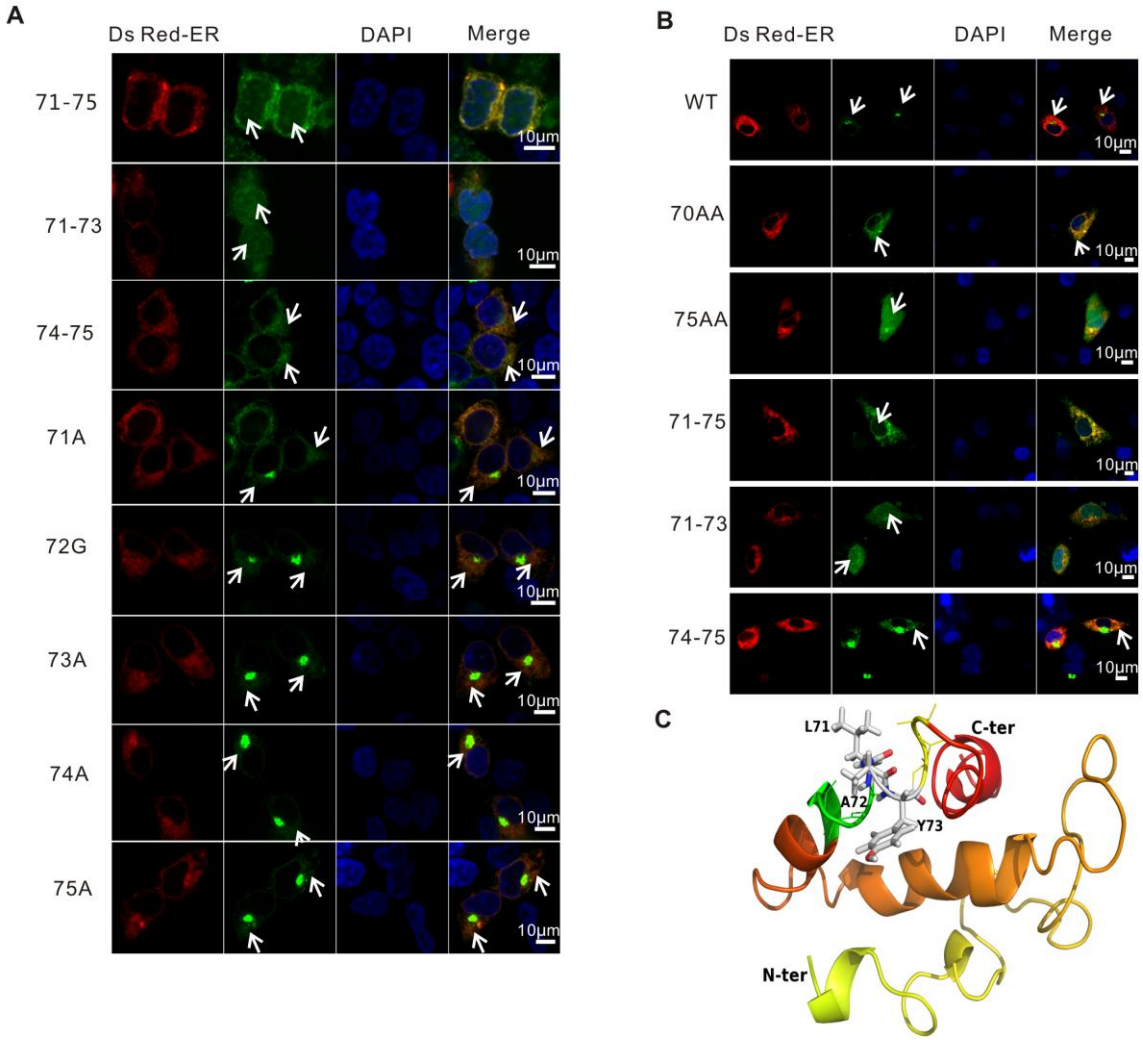

Fig5. *LAY, the ER retention signal of UBIAD1.*

*(**A**) The 71-75 amino acids (LAYRS) in N-terminus was sequentially mutated to AAAAA (71-75), AAARS (71-73), LAYAA (74-75), AAYRS (71A), LGYRS (72G), LAARS (73A) , LAYAS (74A), LAYRA (75A). These mutant UBIAD1-EGFP proteins were expressed in the HEK293T cells (panel 2). (**B**) The wild type (WT) and part of the crucial mutant UBIAD1-EGFP proteins were expressed in bladder carcinoma T24 cells. (**C**) The predicted 3D structure of the UBIAD1 N-terminus using Rosetta3.1*

(http://www.rosettacommons.org/manuals/archive/rosetta3.1_user_guide/).

3.5. The Mutant Protein UBIAD1(RPWS→AAAA) was Only Distributed on the ER

UBIAD1 is first synthesized on the ER and then targeted to the Golgi via anterograde trafficking, as UBIAD1 resides on the endoplasmic reticulum (ER) and the Golgi in HEK293 cells [3]. Mutation of the Golgi retention signal RPWS could change the accumulation of UBIAD1 on the Golgi. To further investigate the partition of UBIAD1 between the Golgi and the ER, cycloheximide was used to block UBIAD1 synthesis (Figure 6). The mutant protein UBIAD1(RPWS→AAAA) was overexpressed in HEK293T cells. Following 20 hours of UBIAD1 transfection, the transfected HEK293T cells were treated with cycloheximide for 0 hours as control (Figure 6, a), 4.5 hours (Figure 6, b), 5 hours (Figure 6, c), 10 hours (Figure 6, d) and 20 hours (Figure 6, e), respectively. In the control group (Figure 6, a), mutant protein UBIAD1 (RPWS→AAAA) seems to only be distributed on the ER and the patch on the Golgi disappeared. After the UBIAD1 synthesis was blocked for 4.5 hours, the distribution of mutant protein UBIAD1 (RPWS→AAAA) was similar to that of the control. However, the green fluorescence on the ER becomes strong or indicating retention of UBIAD1 (RPWS→AAAA) on the ER. After the UBIAD1 synthesis was blocked for 5 hours, 10 hours, 20 hours, the mutant protein was still localized on ER and the green fluorescence has been weakened. It seems to be that the mutant protein has partially been degraded.

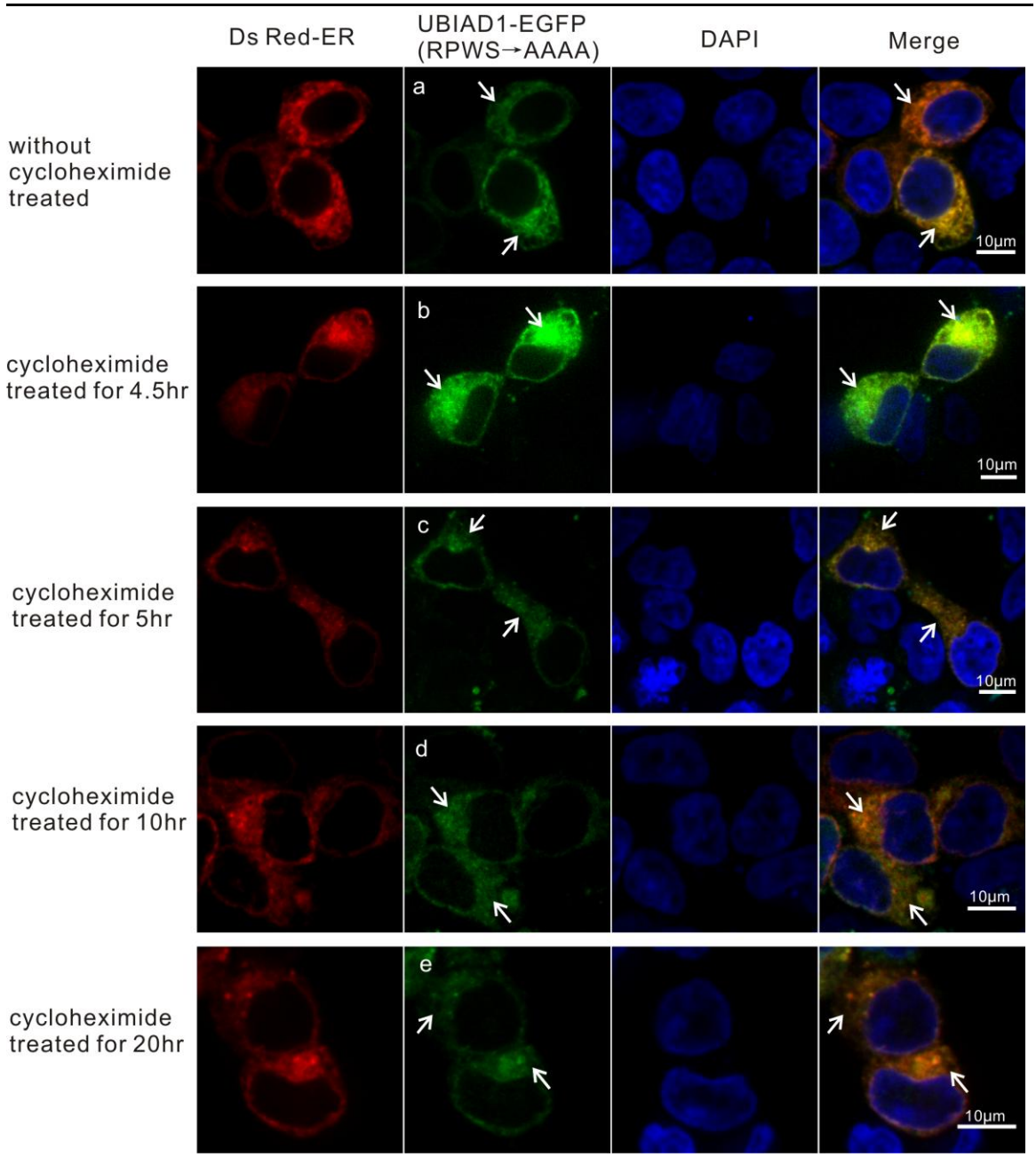

Fig6. *Intracellular trafficking of UBIAD1*

The Golgi retention signal RPWS mutant protein (RPWS→AAAA) was expressed in HEK293T cells. Following 20 hours of mutant UBIAD1 expression, the HEK293T cells were treated with cycloheximide (100ug/mL) for 4.5 hrs (b), 5 hrs (c), 10 hrs (d) and 20 hrs (e), respectively. Nuclei of the HEK293T cells were stained with DAPI.

3.6. Mutation of ER Retention Signal Influences the Function of UBIAD1

Subcellular localization of protein is usually closely related to its physiological function. To explore this connection, we tested the effect of destroying the ER localization of UBIAD1 on its tumor suppressing activity. Bladder carcinoma T24 cells were transfected with wild type UBIAD1 and mutant (LAYRS→AAAAA) UBIAD1. After 48 hours expression, the T24 cells were fixed with 70% ethanol overnight and then stained with PI following wash with PBS. The flow cytometry was used to detect the cell number in different stages of cell circle. We counted the relative number of cells in sub G1 stage, which represents the apoptotic cells. In Figure 7E, the columns show the percentage of cells in sub G1 stage. And the height of the fourth column (the group with mutant UBIAD1 treated) is higher than the third one (the group with wild type UBIAD1 treated), which means that the mutant UBIAD1 (which can not be localized on the ER) can cause more DNA damage resulting from more potential cell apoptosis.

The specialization of organelles for different functions means that each requires a unique composition of small molecules and macromolecules. The basic principle of protein localization is that any protein whose destination is a specific organelle carries within it a short amino acid sequence that constitutes a sorting signal (or targeting signal). Each type of organelle has one or more distinct classes of signals. There are two mechanisms for Golgi-to-ER transport. The best-known one relies on the Arf1 GTPase and COPI coatomer that the retrograde membrane cargo carrying COPI recognition motifs can be retrieved. Usually, the KDEL receptor and Rer1p function as receptors to bind and retrieve escaped ER residents. In our research, we found the new LAY signal and we assumed that the ER retention of UBIAD1 depends on this pathway. The second pathway is Arf1-/COPI- independent and relies on the Rab6 GTPase instead. This pathway appears to involve tubules rather than vesicles and is responsible for cycling of glycosylating enzymes between the Golgi and the ER.

Disrupting the ER localization of UBIAD1 enhances its potential tumor suppressing activity, suggesting that the ER may not be the organelle for its biological function as a tumor suppressor. Since UBIAD1 is a multifunctional disease protein, we should further study its physiological function with specific subcellular localization.

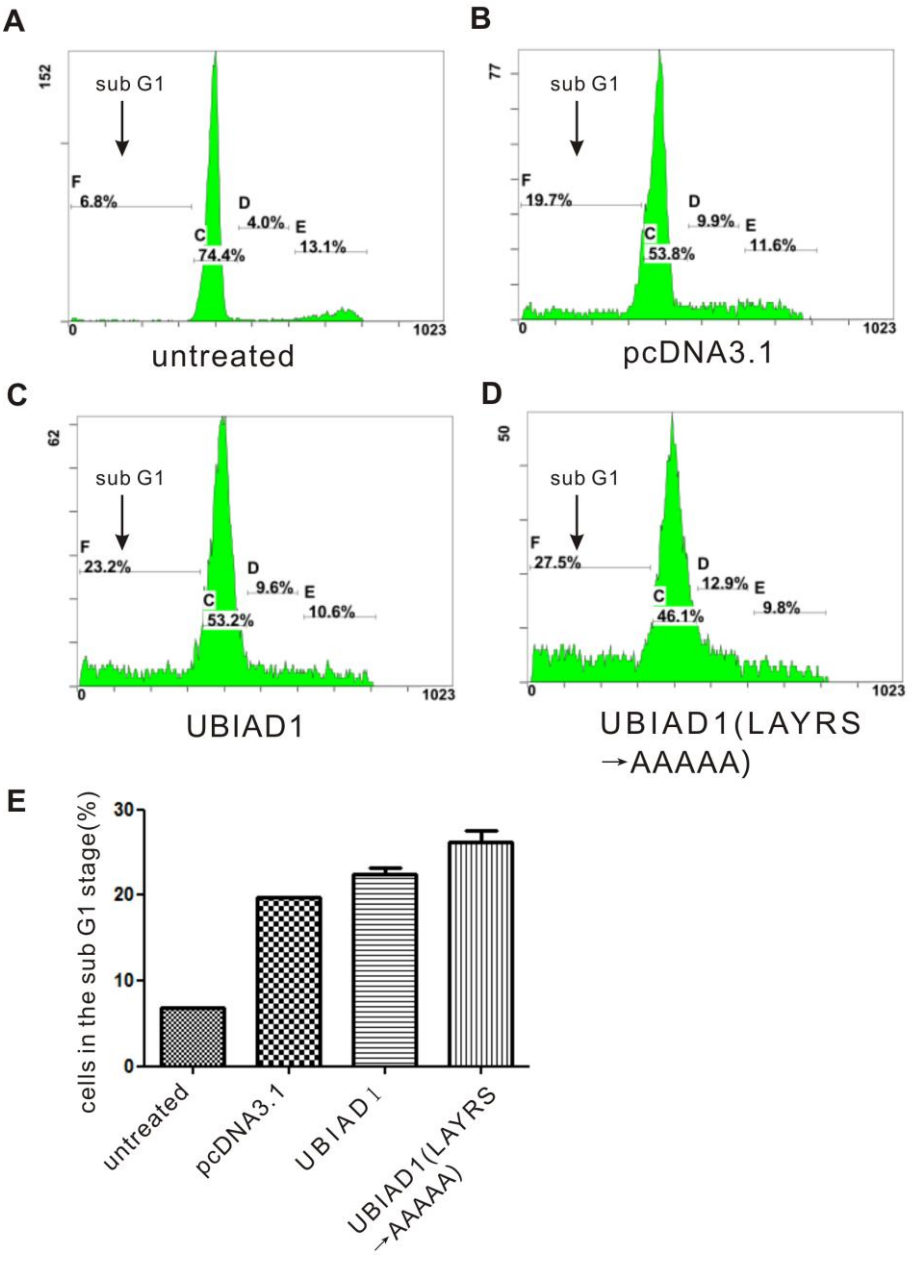

Fig7. *Mutating the ER retention signal of UBIAD1 influences its tumor suppressing activity.*

*The bladder carcinoma T24 cells were treated with wild type and mutant (LAYRS→AAAAA) UBIAD1 respectively, followed by the staining with PI after fixed with 70% ethanol overnight. Cell counts were performed with a flow cytometer. (**A**) Untreated, T24 cells were stained with PI after fixed with 70% ethanol overnight; (**B**) pcDNA3.1, T24 cells were transected with pcDNA3.1 (fixed with 70% ethanol overnight, then stained with PI 48 hr later); (**C**) UBIAD1, T24 cells were transfected with pcDNA3.1-UBIAD1 (fixed with 70% ethanol overnight, then stained with PI 48 hr later); (**D**) UBIAD1 (LAYRS→AAAAA), T24 cells were transfected with (LAYRS→AAAAA) mutant of UBIAD1 (fixed with 70% ethanol overnight, then stained with PI 48 hr later); (**E**) Comparison of cell percentage in the sub G1 stage (F area) between wild type and (LAYRS→AAAAA) mutant UBIAD1 on T24 cells.*

4. CONCLUSION

In conclusion, we showed that UBIAD1 is colocalized with endoplasmic reticulum in HEK293T cells and T24 cells. The potential endoplasmic reticulum retention signal LAY has been identified and the function of UBIAD1 as a tumor suppressor depends on its subcellular localization. This guideline is used for all journals. These are the manuscript preparation guidelines used as a standard template for all journal submissions. Author must follow these instructions while preparing/modifying these guidelines. This guideline is used for all journals. This guideline is used for all journals. These are the manuscript preparation guidelines used as a standard template for all journal submissions. Author must follow these instructions while preparing/modifying these guidelines. This guideline is used for all journals. Our work laid the foundation for further study the function of UBIAD1 on the ER and its physiological meaning.

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REFERENCES

[1] McGarvey T.W., Nguyen T., Tomaszewski J.E., Monson F.C., and Malkowicz S.B., Isolation and characterization of the TERE1 gene, a gene down-regulated intransitional cell carcinoma of the bladder, Oncogene 20: 1042–1051 (2001).

[2] McGarvey T.W., Nguyen T., Puthiyaveettil R., Tomaszewski J.E., and Malkowicz S.B., TERE1, a novel gene affecting growth regulation in prostate carcinoma, Prostate 54: 144–155(2003).

[3] Orr A., Dube MP, Marcadier J., Jiang H., Federico A., et al, Mutations inthe UBIAD1 gene, encoding a potential prenyltransferase, are causal for Schnyder crystalline corneal dystrophy, PLoS One 2: e685(2007).

[4] Yellore V.S., Khan M.A., Bourla N., Rayner S.A., Chen M.C., et al, Identification of mutations in UBIAD1 following exclusion of coding mutations in the chromosome 1p36 locus for Schnyder crystalline corneal dystrophy, MolVis 13: 1777–1782 (2007).

[5] Weiss J.S., Kruth H.S., Kuivaniemi H., Tromp G., White P.S., et al, Mutations in the UBIAD1 gene on chromosome short arm 1, region 36, cause Schnyder crystalline corneal dystrophy, Invest Ophthalmol Vis Sci 48: 5007–5012 (2007).

[6] Nickerson ML, Kostiha B.N., Brandt W., Fredericks W., Xu K.P., et al, UBIAD1 mutation alters a mitochondrial prenyltransferase to cause Schnydercorneal dystrophy, PLoS One 5: e10760 (2010).

[7] McGarvey T.W., Nguyen T.B., Malkowicz S.B., An interaction between apolipoprotein E and TERE1 with a possible association with bladder tumor formation, J Cell Biochem 95:419–428 (2005).

[8] Fredericks W.J., McGarvey T., Wang H., Lal P., Puthiyaveettil R., et al, The Bladder Tumor Suppressor Protein TERE1 (UBIAD1) Modulates Cell Cholesterol: Implications for Tumor Progression, DNA AND Cell Biol 30: 851–864(2011).

[9] Nakagawa K., Hirota Y., Sawada N., Yuge N., Watanabe M., et al, Identification of UBIAD1 as a novel human menaquinone-4 biosynthetic enzyme, Nature 468: 117–121(2010).

[10] Vos M., Esposito G., Edirisinghe J.N., Vilain S., Haddad D.M., et al, VitaminK2 Is a Mitochondrial Electron Carrier That Rescues Pink1 Deficiency, Science336: 1306–1310 (2012).

[11] Mugoni V., Postel R., Catanzaro V., Luca E.D., Turco E., et al, Ubiad1 is an antioxidant enzyme that regulates eNOS activity by CoQ10 synthesis, Cell 152:504–518 (2013).

[12] Xia Y.Z., Wei X., Wu Shimin, Wang Bo, Wang X.M., Hong L., Down-regulation of TERE1/UBIAD1 activated Ras–MAPK signaling and induced cell proliferation, Cell Bio Int Rep 17: art: e00005.doi:00010.01042/CBR20100005 (2010).

[13] Xia Y., Midoum S.Z., Xu Z., Hong L., Heixuedian (heix), a potential melanotic tumor suppressor gene, exhibits specific spatial and temporal expression pattern during drosophila hematopoiesis, Dev. Biol. (2014).

[14] Wang X., Wang D.F., Jing P., Wu Y.G., Xia Y.Z., et al, A novel Golgi retention signal RPWS for tumour suppressor UBIAD1, PLoS One 8: 72015 (2013).

[15] Prakriya M., Feske S., Gwack Y., Srikanth S., Rao A., et al, Orai1 is an essential pore subunit of the CRAC channel, Nature 443: 230–233 (2006).

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